Final Scientific Report

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Project Title: Improving crustacean aquaculture production efficiencies through development of monosex populations using endocrine and molecular manipulations

Investigators		Institutions
Principal Investigator (PI): Abigail Elizur		Dept. Primary Industries & Fisheries and University of the Sunshine Coast
Co-Principal Investigator (Co-PI): Amir Sagi		Dept. life Sciences and the National Inst. for Biotechnology in the Negev, Ben Gurion University
Collaborating Investigators: Gideon Hulata		Dept. Poultry & Aquaculture Sciences, Inst. of Animal Science, Agricultural Research Organization
	Clive Jones	Dept. Primary Industries & Fisheries
	Wayne Knibb	University of the Sunshine Coast

Keywords- Androgenic gland, *Cherax quadricarinatus*, *Penaeus monodon*, insulin-like gene, sex differentiation, RNA interference

Abbreviations commonly used in the report, in alphabetical order: AG- Androgenic gland; BGU - Ben Gurion University of the Negev; *Cq-IAG- C. quadricarinatus* insulin-like AG factor; DPI&F- Department of Primary Industries and Fisheries; dsRNA- double-stranded RNA; *Pm-IAG- P. monodon* insulin-like AG factor; qRT-PCR- quantitative real time RT-PCR; RNAi- RNA interference

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Signature	**Signature**
Principal Investigator	**Authorizing Official, Principal Institution**
Signature	**Signature**
Co-Principal Investigator	**Authorizing Official, Co-Principal Institution**

Eti Hecht-Vazana
Coordinator,Research Liaison Section
Research & Development Authority

Research & Development Authority
Ben-Gurion University of the Negev
P.O. Box 635, Beer-Sheva 84105,Israel

Abstract

Background

Most of Australian prawn aquaculture production is based on *P. monodon*. However, the Australian industry is under intense competition from lower priced overseas imports. The availability of all-female monosex populations, by virtue of their large size and associated premium prize, will offer competitive advantage to the industry which desperately needs to counteract competitors within this market. As for the redclaw production in Israel, although it is at its infancy, the growers realized that the production of males is extremely advantageous and that such management strategy will change the economic assumptions and performances of this aquaculture to attract many more growers.

Original objectives (as in original proposal)

1. Investigating the sex inheritance mechanism in the tiger prawn.
2. Identification of genes expressed uniquely in the androgenic gland (AG) of prawns and crayfish. The above genes and/or their products will be used to localize the AG in the prawn and manipulate the AG activity in both species.
3. Production of monosex populations through AG manipulation. In the prawn, production of all-female populations and in the crayfish, all-male populations.

Achievements

In the crayfish, the AG cDNA library was further screened and a third AG specific transcript, designated *Cq-AG3*, had been identified. Simultaneously the two AG specific genes, which were previously identified, were further characterized. Tissue specificity of one of those genes, termed *Cq-AG2*, was demonstrated by northern blot hybridization and RNA *in-situ* hybridization. Bioinformatics prediction, which suggested a 42 amino acid long signal anchor at the N-terminus of the deduced Cq-AG2, was confirmed by immunolocalization of a recombinant protein. *Cq-IAG*'s functionality was demonstrated by dsRNA *in-vivo* injections to intersex crayfish. *Cq-IAG* silencing induced dramatic sex-related alterations, including male feature feminization, reduced sperm production, extensive testicular apoptosis, induction of the *vitellogenin* gene expression and accumulation of yolk proteins in the ovaries. In the prawn, the AG was identified and a cDNA library was created. The putative *P. monodon* AG hormone encoding gene (*Pm-IAG*) was identified, isolated and characterized for time of expression and histological localization. Implantation of the AG into prawn post larvae (PL) and juveniles resulted in phenotypic transformation which included the appearance of *appendix masculina* and enlarged *petasma*. The transformation however did not result in sex change or the creation of neo males thus the population genetics stage to be executed with Prof. Hulata did not materialized. Repeated AG implantation is currently being trialed.

Major conclusions and Implications, both scientific and agricultural

Cq-IAG's involvement in male sexual differentiation had been demonstrated and it is strongly suggested that this gene encodes an AG hormone in this crayfish. A thorough screening of the AG cDNA library shows *Cq-IAG* is the prominent transcript within the library. However, the

identification of two additional transcripts hints that *Cq-IAG* is not the only gene mediating the AG effects. The successful gene silencing of *Cq-IAG*, if performed at earlier developmental stages, might accomplish full and functional sex reversal which will enable the production of all-male crayfish populations. *Pm-IAG* is likely to play a similar role in prawns. It is possible that repeated administration of the AG into prawn will lead to the desired full sex reversal, so that WZ neo males, crossed with WZ females can result in WW females, which will form the basis for monosex all-female population.

Achievements

C. quadricarinatus AG-specific genes

In the crayfish, the existing cDNA library was further screened. In order to eliminate ESTs representing the identified *Cq-IAG*, which is most prominent in the AG transcriptome, we used the colony hybridization method using a *Cq-IAG*-based probe. Out of 508 colonies which were examined by colony hybridization, 115 were considered positive for *Cq-IAG* (Fig. 1A).

Totally, 477 ESTs of the AG cDNA library have been assembled into contigs and singlets, representing 87 putative genes. As shown in Fig. 1B, three AG uniquely expressed genes comprise about 30% of the above mentioned library, with *Cq-IAG*, as the most prominent transcript with a slice of approx. 25%, *Cq-AG2* (5%) and *Cq-AG3* (0.4%). When analyzing the remaining sequences, more than 50% of the assembled products showed no significant similarity (E value>0.01) to any known sequence in the database, however, RT-PCR indicate that none of those sequences is an AG specifically expressed gene. The remaining sequences were associated with various cellular-related functions.

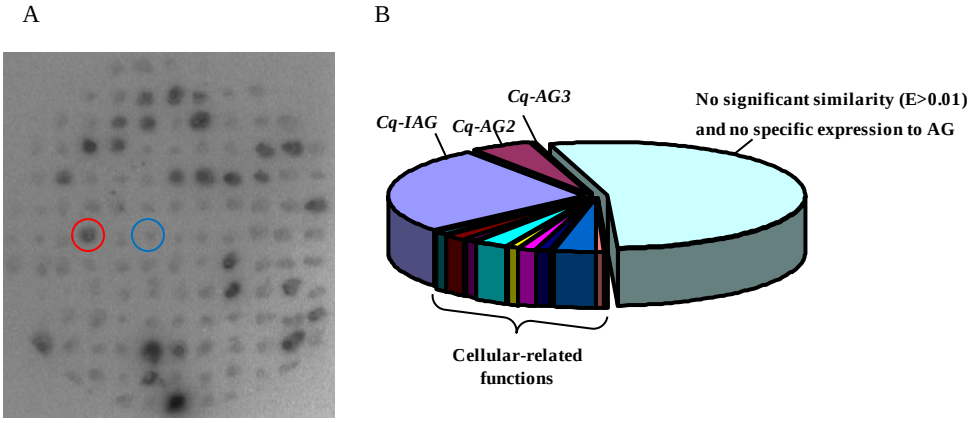

Figure 1: *Colony hybridization and full gene annotation of the C. quadricarinatus AG cDNA subtractive library*

Further characterization of *Cq-AG2* by northern blot analysis revealed a transcript of about 920 b, which has been observed only in the lane containing RNA from the AG (Fig. 2A). *In-situ* hybridization has also confirmed that *Cq-AG2* specific expression is restricted to the AG (Fig. 2B). A specific signal in the AG cells was clearly observed only when using the antisense probe.

Later on, *Cq-AG2* was fully sequenced and its open reading frame of 567 b encodes a deduced 189 amino acid product. Bioinformatics analysis (CBS Prediction Servers, http://www.cbs.dtu.dk/services), predicts a putative 42 amino acid-long signal anchor peptide at the N-terminus (Signal anchor probability: 0.912). An experiment examining the role of this putative signal anchor was performed in SR+ Schneider cells from *Drosophila*. Localizations of recombinant Cq-AG2::GFP fusion proteins were determined by using an anti-GFP antibody. When expressing the full sequence of *Cq-AG2* fused to *GFP*, green fluorescence was observed in spots while when expressing the truncated *Cq-AG2* (lacking 42 amino acids of the N-terminus) fused to *GFP*, fluorescence was evenly spread throughout the cell (Fig. 2C).

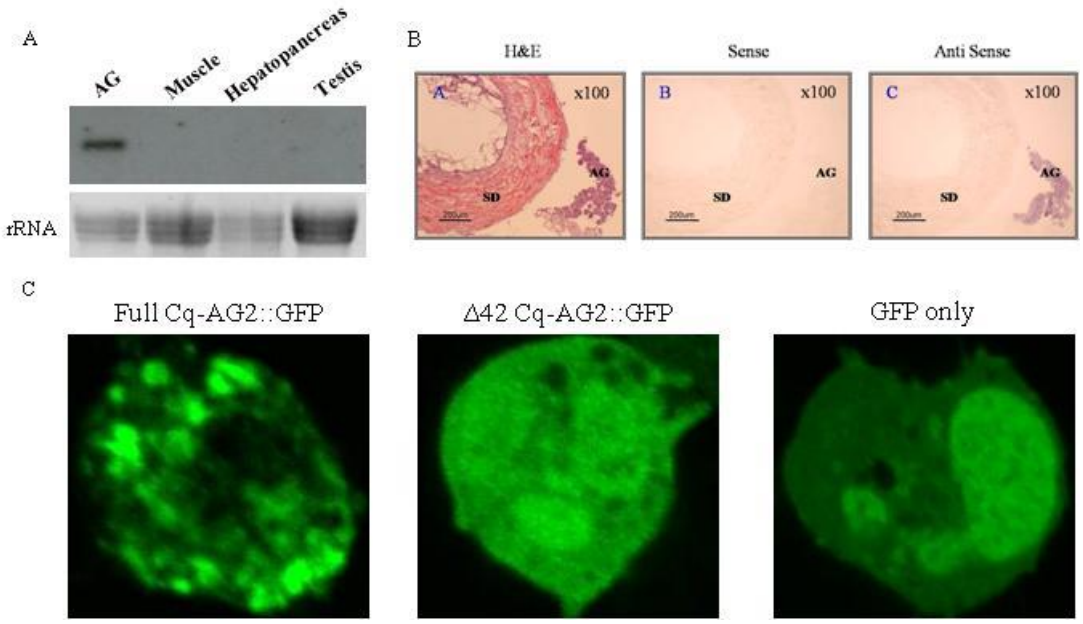

Figure 2: *Conformation of Cq-AG2 tissue specificity as demonstrated by northern blot hybridization (A) and RNA in-situ hybridization (B). Differential localizations of recombinant Cq-AG2::GFP fusion proteins in Drosophila Schneider cells*

Sexual shift through gene silencing

In *C. quadricarinatus*, sexual plasticity is exhibited by intersex individuals in the form of an active male reproductive system and male secondary sex characters along with a constantly arrested ovary. We exploited this intersexuality to follow changes caused by single gene silencing, which was accomplished via dsRNA injection (see Appendix A). *Cq-IAG* silencing induced

dramatic sex-related alterations. Upon silencing, AG cells hypertrophied (Appendix A, Fig. 6), possibly to compensate for low hormone levels, given the poor production of the insulin-like hormone, as revealed by immunohistochemistry (Appendix A, Fig. 7). Male-structured pleopods were feminized and exhibited egg-bearing morphologies related to maternal care (Appendix A, Fig. 3). The silenced male reproductive system produced less sperm (Appendix, Fig. 5A), and extensive testicular apoptosis was observed (Appendix A, Fig. 5B). Simultaneously, there was a significant increase in ovarian size (Appendix A, Fig. 4), accompanied by expression of the *vitellogenin* gene (Appendix A, Fig. 8) and accumulation of yolk proteins in the developing oocytes (Appendix A, Fig. 5A).

P. monodon AG

The exact- location of the AG in prawns was not previously determined. Through extensive histological studies, we have been able to identify the AG in the black tiger prawn, *P. monodon* (Fig. 3)

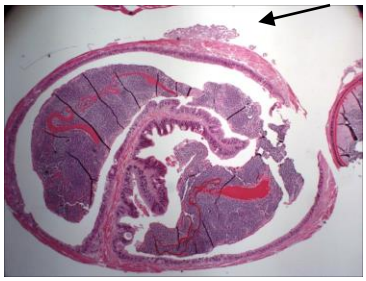

Figure 3: *A histological section showing AG cells adjoining the sperm duct, in the male P. monodon*

A *P. mondon* AG cDNA library was created and sequencing of 96 clones resulted in 9 contigs and 50 singlets, thus representing 59 putative genes. Among those was the *Pm-IAG*, the identification of which was based on sequence homology with the *Cq-IAG*. Though there was a high degree of divergence between the sequences, the cysteine backbone conservation, typical of Insulin superfamily of genes, provided necessary clues to the finding of *Pm-IAG*. In addition, several other sequences were identified, including profiling, which is involved in insulin receptor signaling pathway, prefolding, which is involved in protein folding and is part of the prefolding complex as well as other housekeeping genes involved in general cell functions. Twenty nine out of the 59 transcripts were not assigned gene ontological terms (Fig. 4).

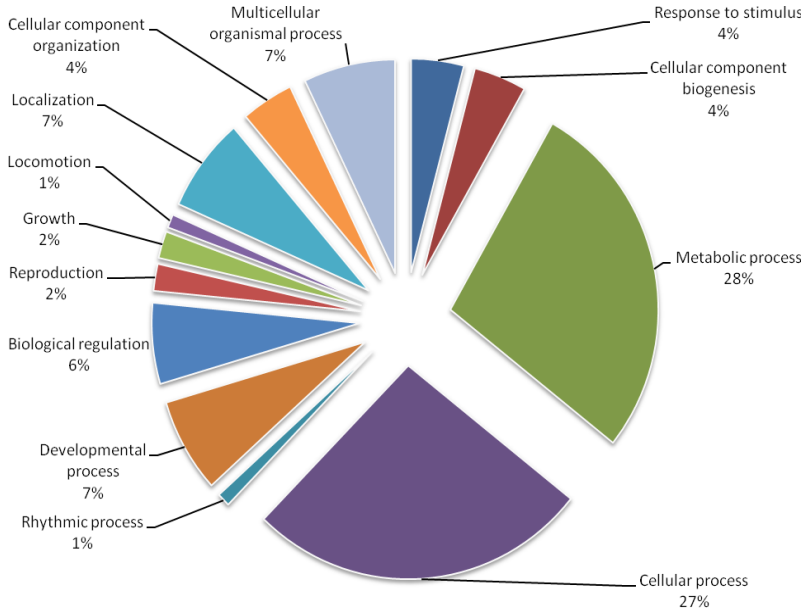

Figure 4: *Blast2Go annotation results of the P. monodon AG cDNA library*

Molecular characterization of the gene *Pm-IAG* revealed a deduced coding sequence of 531 nucleotide bases and a mature translation product (Pm-IAG) of 76 amino acids. The tissue specificity of *Pm-IAG* is presented in appendix B (Fig.1 and 2). The time of expression of *Pm-IAG* which was examined by RT-PCR, was found to be indeed expressed from PL45.

Protein expression in the AG was examined using 2D gel analysis, comparing between AG tissue and sperm duct tissue. The protein spots in the 19 kDa range are in agreement with the estimated molecular weight of the Pm-IAG pre-pro-peptide (Fig. 5).

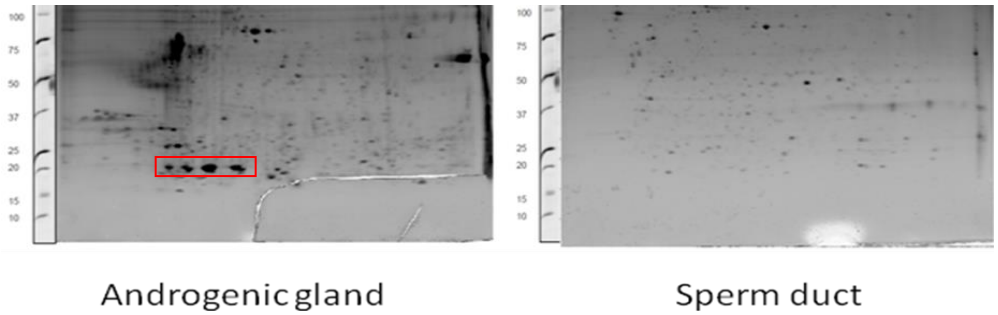

Figure 5: *2D gel images reveal the differential protein spots in the AG when compared to sperm duct. Note the protein spots in the 19 kDa range of the AG tissue*

During the second year of the project, the sex inheritance of prawns was determined by another group (Staelens et al, 2008), and was found to be, as in the crayfish, ZZ/WZ. Following that, there was a need to masculinize females to obtain WW females for the creation of all-female populations. The *P. monodon* AG was implanted into young females and a phenotypic

transformation has occurred, resulting in the appearance of an *appendix masculina* and enlarged *petasma*. None of the transformed females however have developed into functional males (Fig. 6).

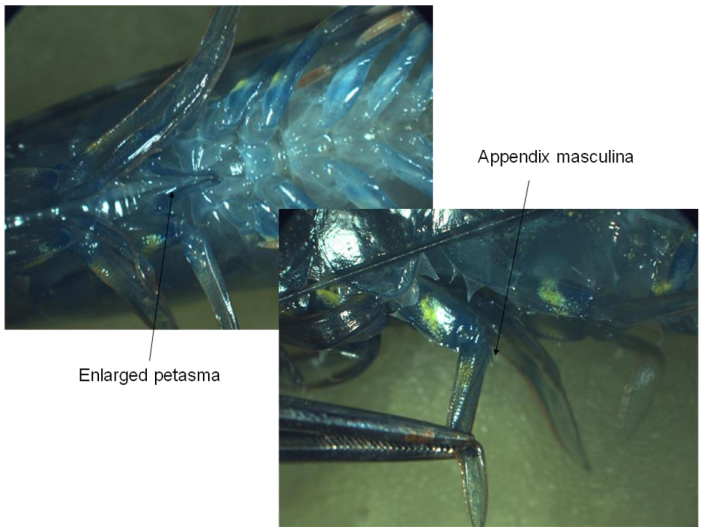

Figure 6: *Transforming females showing phenotypic male characteristics following AG implantation*

Histological studies, however showed no internal changes with female prawns showing ovarian development including stages of lipid deposition thus indicating continued vitellogenesis (Fig. 7)

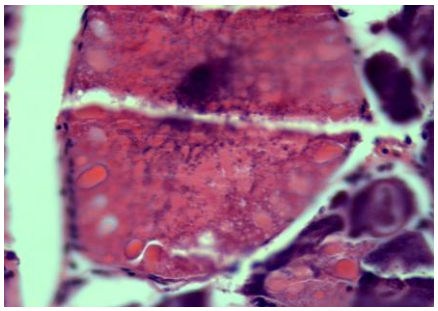

Figure 7: *Ovarian cell showing lipid globules*

Details of cooperation

The project objectives were promoted as a result of the cooperation between the groups, which has been continuous through electronic media approximately once a week. The BGU team provided DPI&F sequences specific to the AG from the screening of its subtractive cDNA library of the redclaw crayfish *C. quadricarinatus* and those were used to try to isolate the orthologous genes from prawns which lead to the identification of *Pm-IAG*. The DPI&F team

provided protocols and professional advices for the colony hybridization method. Prof. Abigail Elizur visited the BGU laboratory on March 2007 and was updated with the BGU state of research and localization of the AG was demonstrated. Surgical manipulation of the AG has been demonstrated and practiced. This information was critical for the successful identification of the prawn AG at DPI&F. This visit was also used to discuss and finalize the program and mode of collaboration in detail including a discussion held at the Marine Biotechnology conference with Prof. Gideon Hulata. During her visit at the BGU laboratory, Prof. Elizur gave a seminar introducing the state of research at the DPI&F laboratory. During the second year of the project a second meeting was held at BGU. In this meeting both sides were fully updated with current advances in the collaborating team. Also, in this meeting, initial arrangements for a student-exchange program were made. This program was executed with the travel of Mr. Ohad Rosen from Prof. Amir Sagi's laboratory to Prof. Elizur's laboratory for a research period of 3 months. This visit included investigation of up-stream genomic regulation of *Cq-IAG* and *Cq-AG2*. In this study, we attempted for the first time in crustaceans, to isolate sequences outside the transcript (cDNA) frame using genomic DNA. Our main target was full characterization of the AG genes in the genomic sequence level, ultimately isolating the promoter and other expression controlling elements (positive and negative). Thus far, we have succeeded in isolating 2 intron sequences (1 in each gene) which enables a first glimpse at the genome of that species, and provides the first genomic data of AG uniquely expressed genes.

Followed by *Pm-IAG* identification, an *in situ* hybridization experiment was done at BGU to confirm the localization of *Pm-IAG* expression. In addition, a 3D model of Pm-IAG was suggested by the BGU team.

Appendix

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